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In situ use of bivalves and passive samplers to reveal water contamination by microcystins along a freshwater-marine continuum in France

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Abstract :

Cyanobacteria are a potential threat to aquatic ecosystems and human health because of their ability to produce cyanotoxins, such as microcystins (MCs). MCs are regularly monitored in fresh waters, but rarely in estuarine and marine waters despite the possibility of their downstream export. Over a period of two years, we monthly analysed intracellular (in phytoplankton) and extracellular (dissolved in water) MCs at five stations along a river continuum from a freshwater reservoir with ongoing cyanobacterial blooms to the coast of Brittany, France. MCs were quantified using two integrative samplers placed at each site: solid phase adsorption toxin tracking (SPATT) samplers for collecting extracellular MCs and caged mussels (Anodonta anatina and Mytilus edulis) filter-feeding on MC-producing cyanobacteria. The MC transfer was demonstrated each year during five months at estuarine sites and sporadically at the marine outlet. SPATT samplers integrated extracellular MCs, notably at low environmental concentrations (0.2 µg/L) and with the same variant profile as in water. The mussel A. anatina highlighted the presence of MCs including at intracellular concentrations below 1 µg/L. M. edulis more efficiently revealed the MC transfer at estuarine sites than punctual water samplings. Bivalves showed the same MC variant profile as phytoplankton samples, but with differential accumulation capacities between the variants and the two species. Using SPATT or bivalves can give a more accurate assessment of the contamination level of a freshwater-marine continuum, in which the MC transfer can be episodic. MC content in M. edulis represents a potent threat to human health if considering updated French guideline values, and particularly the total (free and protein-bound) MC content, highlighting the necessity to include cyanotoxins in the monitoring of seafood originating from estuarine areas.

Graphical abstract

Highlights

► MC transfer along a freshwater-marine continuum was recurrent over 2 years. ► SPATTs efficiently integrated extracellular MC in fresh and estuarine waters. ► Bivalves efficiently integrated and revealed environmental intracellular MC. ► MC content in *M. edulis* represented a potential threat for human health.

Keywords : cyanotoxins, biosurvey, molluscs, solid phase adsorption toxin tracking (SPATT)

Introduction

Proliferations of freshwater cyanobacteria are a source of growing concern because of the ecological and economical disturbances they create, as well as the significant impact of their toxins on animal and human health (for reviews: Wood et al., 2016; Svirčev et al., 2017). The most frequent cyanotoxins found in freshwater ecosystems are the MCs, among which 246 variants have been described (Spoof and Arnaud, 2017). MCs are mostly within cyanobacteria cells (intracellular) during the cyanobacterial bloom but are released into the water as free or adsorbed on particles (extracellular) when blooms collapse. MCs are mainly hepatotoxic, but were also recently recognised as neurotoxic and reprotoxic (Buratti et al., 2017). Provisional MC thresholds (guideline values) of 1 and 12 μg/L for lifetime and short-term drinking-water respectively have been established by the World Health Organization (WHO) for the widespread and most often studied MC-LR variant (WHO, 2020). The WHO also proposed a provisional upper limit for recreational water of 24 μg/L and a lifetime tolerable daily intake (TDI) of 40 ng/day/kg of body weight for MC-LR.

Most studies on cyanobacteria and cyanotoxin occurrence were conducted in freshwater ecosystems, but intermittent cyanobacteria and MC transfer during a freshwater discharge into the coastal environment were recently reported in California (Gibble and Kudela, 2014), Italy (De Pace et al, 2014), Japan (Umehara et al, 2015), Washington state (Preece et al., 2015; 2017), Korea (Kim et al., 2019), France (Bormans et al., 2019), Lithuania (Overlinge et al., 2020) and Uruguay (Kruk et al., 2021). The resulting MC contamination of marine organisms such as molluscs, crustaceans, fish and even sea otters, has been reported in several countries (Lehman et al, 2010; Miller et al, 2010; Gibble et al, 2016; Tatters et al. 2017; Peacock et al., 2018; Kim et al., 2019), and could have a large health and economic impact. However, the contamination level in organisms consumed by humans may have been underestimated

because only the free (not protein-bound) fraction of MC accumulation is routinely quantified in tissues. After ingestion by organisms and distribution in target cells, MCs either remain free or covalently bind to protein phosphatases (PPases) and proteins containing cystein group (Campos and Vasconcelos, 2010). The protein-bound fraction of MCs has been demonstrated to be predominant in animal tissues and is slowly eliminated (Lance et al., 2010; Lepoutre et al., 2020). MC-cysteine conjugates are still toxic but less so than free MCs (Kondo et al., 1992); the toxicity of MCs bound to PPases has not been determined. The bioavailability and bioaccessibility of protein-bound MCs, that could become free during the digestion process in consumers is also unknown (Mohamed et al., 2018). In the context of increasing average global temperature and periodic droughts and floods, the occurrence of cyanobacterial blooms may become more frequent and intense in the future (Paerl and Paul, 2020), as may cyanotoxin transfer in estuarine zones and marine coastal areas. It may therefore be useful to evaluate the free and total (free plus protein-bound) MC content in seafood from estuarine zones.

The present study represents the second part of a project on cyanobacteria and cyanotoxin transfer along a freshwater-marine continuum in Brittany, France (Bormans et al., 2019, 2020), during which only MCs were found in phytoplankton and water samples, while cylindrospermopsin, anatoxins, and nodularin were absent. The occurrence of MC-producing cyanobacteria in water samples can be difficult to assess because of the spatial (vertical and horizontal migrations) and temporal (rapid proliferation and collapse) dynamics of cyanobacteria populations (Deng et al., 2016; Salmaso et al., 2017). The episodic occurrence of cyanotoxins in marine waters and the effects of tidal water dilution may both influence the reliability and relevance of intermittent water samplings. The use of monitoring devices that integrate cyanotoxins over time may overcome this issue. Passive integrators like SPATT have been developed, but their use is limited because the samplers only adsorb extracellular cyanotoxins and they are subject to rapid clogging and saturation, which shortens their useful

life (Kudela, 2011). Bivalves, filter-feeders with high filtration rates, have been proposed as tools for detecting the presence of MC-producing cyanobacteria in water (Preece et al., 2015; Lauceri et al., 2017; Lepoutre et al., 2020). Bivalves accumulate MCs primarily in the digestive gland, mainly after ingestion of MC-producing cyanobacteria and to a lesser extent through the filtration of dissolved toxins (Ferrão-Filho and Kozlowsky-Suzuki, 2011; Gkelis et al., 2006; Camacho et al., 2021).

In this study, five stations along a freshwater-marine continuum were sampled monthly over two years, and weekly during proliferation periods. The freshwater mussel *Anodonta anatina* was caged at two upstream freshwater sites exposed to intense annual cyanobacterial blooms. The marine mussel *Mytilus edulis* was caged at two estuarine sites and also sampled at a most marine site. Nine MC variants (MC-LR, dmMC-LR, MC-RR, dmMC-RR, MC-YR, MC-LA, MC-LY, MC-LW, MC-LF) were measured in phytoplankton biomass (intracellular), in filtered water (extracellular), in SPATT samplers (extracellular adsorbed on membranes) and in tissues of mussels (free accumulation fraction). Total (free plus protein-bound) MCs were also determined in some mussel tissues. This study aimed at increasing knowledge about: i) the effectiveness of SPATT samplers as an integrative tools of extracellular MCs, notably at low concentrations and in salt water, ii) the value of freshwater and marine bivalves as bioindicators of the presence of MC-producing cyanobacteria in aquatic systems compared to phytoplankton samplings, iii) the potential concern for human food safety by comparing the levels of MC contamination in marine bivalves with the guidelines from the WHO and French policy makers.

2. Materials and methods

2.1. Study sites

The study site was located in the Morbihan (Brittany, France) along a continuum of moderate length (about 8 km), from a freshwater reservoir (Pen Mur) to the estuary and the marine outlet (Pen Lan harbour), through the St Eloi river. The reservoir undergoes annual intense cyanobacterial blooms dominated by the genus *Microcystis*, and freshwater discharges into the estuary have been highlighted (Bormans et al. 2019). Field investigations were performed at five stations: (1) F1 reservoir (freshwater), (2) F2 downstream river (freshwater), (3) E1 brackish downstream river (estuarine), (4) E2 harbour near the mouth of the river (estuarine), and (5) M, the foreshore zone (marine) close to a shellfish farming area (**Fig.1**.).

2.2. Caging of bivalves

A. anatina were collected at site F2 four months before starting caging, sized and placed by groups of 20 at 14 ± 2°C in aerated tanks containing 25 L of Cristalline® water (Saint Yorre, France). *M. edulis* were collected during winter 2016 at the M site, and placed by groups of 50 at 8 ± 2°C in aerated tanks containing 20 L of sea water. During the depuration period, both bivalve species were fed twice a week with 3.7 x 10^7 cells of *Chlorella vulgaris* (Greensea, Mèze, France) / individual / day. The caging of bivalves was done from May 2016 to November 2017. *A. anatina* were distributed per groups of 60 in 3 mm mesh polyethylene cages (40 x 40 x 60 cm) immersed at 1-meter depth at the F1 and F2 sites (two cages per site). *M. edulis* were distributed per groups of 100 in 3 mm mesh polyethylene cages (15 x 30 x 5 cm) immersed at E1 and E2 sites (three cages per site) at a depth ensuring an immersion in the freshwater flow at low tide. Due to the technical impossibility of placing cages at the marine site M, *M. edulis* were sampled in a wild population attached to a buoy.

2.3. Passive sampler devices (SPATT): design and deployment

SPATT samplers were prepared using a 68 mm embroidery frame (Singer, Nantes, France). Two resins were tested, HP-20 and Strata-X, in 500 mL of fresh and salt waters with MC-YR (100 ng/L), MC-RR (412 ng/L) and MC-LR (808 ng/L) under agitation at 30 rpm. Absorption yield were higher with HP-20 resins compared to Strata-X resins in fresh waters and equivalent in marine waters. HP-20 resin was then chosen and 3 g of resin were placed between two layers of a 30 µm nylon mesh (Mougel, France), and clamped in an embroidery frame. Before deployment, the SPATT samplers were soaked for 3 h in methanol to activate the resin and rinsed twice with deionized water to remove methanol residues (Zendong et al., 2014). Three SPATT samplers were deployed at each of the five sites from July to October 2017, with sampling and renewal of samplers every ten days.

2.4. Water, bivalve and SPATT sampling and processing

Water and bivalve samples were obtained once a month from May 2016 to November 2017, twice per month during the proliferation period, and weekly from mid-June to early July 2017 due to a heavy cyanobacterial bloom. At estuarine and marine sites, samplings were performed within one hour of low tide to maximize the freshwater discharge and minimize tidal dilution. Water samples (500 mL) were filtered using Cyclopore track-etched membranes (1 µm pore size, Whatman, Maidstone, UK). The phytoplankton biomass and the filtrate were respectively used for analysis of intracellular and extracellular MCs. The SPATT samplers were retrieved and rinsed with seawater. Water samples for November 2016 to April 2017 were not analysed. MC concentrations were measured in five pools of six digestive glands of *M. edulis*, and six digestive glands of *A. anatina*. On specific dates, MC concentrations were measured in whole *M. edulis* to evaluate the MC ratio in the digestive

gland versus the body. After dissection, the fresh tissues were washed with Milli-Q water. All samples were stored at -80°C until chemical analysis.

2.5. Chemical analysis of MCs by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

2.5.1. Analysis of MCs in water, SPATT samplers, phytoplankton and bivalves (free accumulation fraction)

The nine MC variants (MC-LR, dmMC-LR, MC-RR, dmMC-RR, MC-YR, MC-LA, MC-LY, MC-LW, MC-LF, Novakits, France) were measured in phytoplankton, water, mussels and SPATT samplers. Extracellular MCs were purified by solid-phase extraction on 200 mg C18 cartridges (Bond Elut, Agilent). After conditioning with 10 ml of MeOH and 10 ml of Milli-Q water, 500 ml of filtrate was loaded, rinsed with 4 ml of MeOH/Milli-Q water (5:95, v/v), and eluted with 2 ml of MeOH. The eluate was dried under a nitrogen stream, and dissolved in 0.5 mL of MeOH. The phytoplankton biomass was ground in a bead mill with 250 mg of glass beads (0.15-0.25 mm) in 1 mL of MeOH at 30 Hz for 30 min, and centrifuged at 15,000 g for 5 min at 4°C. The supernatant was kept at 4°C and another millilitre of MeOH was added to the pellet to repeat the extraction. Then both supernatants were pooled together, and 400 µL was filtered through a membrane filter (0.2 µm) and centrifuged 1 min at 14,000 g before analysis. The concentrations of extracellular and of intracellular MCs were expressed as µg/L of water sampled *in situ*. For analysis of free MCs accumulated in bivalves, 200 mg tissue samples were homogenized using an Ultra-Turrax homogenizer for 30 sec at 15,000 tr/min. Extraction was performed with 750 µL of 100% MeOH. Samples were ground with 250 mg of glass beads (0.15-0.25 mm) at 30 Hz for 3 min, and centrifuged at 15,000 g for 5 min at 4°C. The supernatant was kept at 4°C and 750 µL of 100% MeOH was added to the pellet to

repeat the extraction. Both supernatants were pooled together. All samples were spin-filtered through 0.2 µm membranes by centrifugation for 10 min at 14,000 g before LC-MS/MS analysis. Free MC contents in mussel tissues were expressed as ng/g of fresh weight (FW). The SPATT samplers were extracted according to Zendong et al. (2014). Briefly, HP-20 resins were rinsed twice in 500 mL deionized water, transferred into empty polypropylene columns and eluted drop-wise with 10 ml of MeOH (2 x 5 ml). The extracts were evaporated at 40°C under nitrogen stream. The dry residue was reconstituted in 500 µL of MeOH, filtered through Nanosep MF centrifugal filters 0.2 µm and transferred into LC vials. The MC concentrations in SPATT were expressed as ng/g of resin/day of deployment. The instrument settings for LC-MS/MS analysis of MCs in water, SPATT, cyanobacteria and bivalves, their specific transitions, as well as their LOD and LOQ, are presented in supplementary material (**Table S1**). The quantification of MCs was done using an external calibration curve with six concentrations of standards dissolved in MeOH. Chromatograms of MC variants are shown in figure S1.

2.5.2. Analysis of total (free plus protein-bound) MCs in *A. anatina* and *M. edulis*

The total MC content in bivalve digestive glands was determined by quantification of the erythro-3-methoxy-2-methyl-4-phenylbutyric acid (MMPB, Tokyo Chemical Industry, Japan), a product of MC oxidation. The method was adapted from Neffling et al. (2010), with modification of the extraction step: two oxidant concentrations, 0.1 M and 0.025 M $KMNO_4/INAO_4^-$ were tested, and extractions were done using SPE cartridges (HLB 3 cc, 60 mg, Waters, MA, USA) versus liquid-liquid extraction with AcOEt. Based on the results, the 0.025 M oxidant solution and liquid-liquid extraction were chosen as most efficient. Fresh 100 mg tissue samples were homogenized using an Ultra-Turrax instrument and digested in Sorensen's buffer (0.1 M, pH 7.5) with 20 µL of trypsin (25 g/L) at 37°C with stirring at 650 rpm for protein hydrolysis. The oxidation was performed with 5 mL of 0.025 M

$KMNO_4/INAO_4^-$ (0.1 M Sorensen's, pH 8.5) for 3 h with mild stirring at 37°C. The reaction was quenched with sodium bisulfite ($NaHSO_3$) until the liquid became transparent, and pH was adjusted to 3 with 10% sulfuric acid. The liquid-liquid extraction was performed with 5 mL of AcOEt, and samples were centrifuged 5 min at 2500 g and 4° C. The procedure was repeated and both supernatants were pooled, dried under nitrogen at 35°C, resuspended in 1 mL of 40% MeOH and filtered (0.2 µm). The quantification of MMPB was performed by spiking blank samples with 1, 5, 10, 25, 50, 75, 100 ng of MMPB to generate an external calibration curve. The percent recovery of total MCs in mussel tissues was determined by spiking blank tissues of each species with MC-LR (10.1 µg/mL) before oxidation, with MMPB (1 µg/mL) after oxidation and before liquid-liquid extraction, and with MMPB (1 µg/mL) after extraction and drying. The recoveries were 26% from oxidation, 81% from extraction, and 89% from ionisation, with a global percent recovery of 19%. Total MC concentrations in the whole mussels (ng/g FW) were calculated based on the global percent recovery of the method and on the proportions of MCs in the digestive glands versus in the whole mussels established on *M. edulis* during this study (93%, data not shown) and on *A. anatina* (89%, Lepoutre et al., 2020). Instrumental conditions for LC-MS/MS analysis of the MMPB and its specific transition are presented in supplementary material (**Table S1**).

2.6. Statistics

Statistical analyses were performed with Statistica (Version 8.0.360.0, Statsoft, Tulsa, USA, 2007). The normality was verified by Shapiro-Wilk test, and the homogeneity of variances was studied with a Levene test. The comparison of two independent samples was done with Mann-Whitney tests and the comparison of multiple independent samples was done with Kruskal-Wallis tests. A chi-squared test was used to compare percentages of each MC variant to the total MC concentration in water samples, SPATT samplers, phytoplankton, and mussel tissues. The significance threshold was set at $p < 0.05$.

3. Results and Discussion

3.1. Efficiency of SPATT samplers for adsorbing extracellular MCs from water

Extracellular MCs were found at low concentrations in water from July to October 2017, with a maximum of 0.65 µg/L in the freshwater reservoir (**Fig. 2).** The MC concentrations extracted from SPATT membranes (expressed as µg MCs/g of resin/day) show that SPATT samplers continuously revealed the presence of extracellular MCs, including for low environmental concentrations (0.12 µg/L) and in saltwater. The use of passive SPATT samplers allowed integration of extracellular MCs and to follow their transfer to the foreshore. The qualitative extracellular MC profile at freshwater sites (**Fig. 3A**) showed a dominance of MC-LR (mean percentage of MC concentration, %var, of 51-62% at F1 and F2 in water, and 47-49% on SPATT samplers), followed by MC-RR (%var of 26-35% at F1 and F2 in water, and 36-42% on SPATT samplers), and MC-YR (%var of 10% at F1 and F2 in water, and 7-11% on SPATT samplers). The MC variant profiles were similar between filtered waters and SPATT samplers, showing that SPATT membranes adsorbed all MC variants equally. This was also true at estuarine and marine sites, where the proportions of MC variants were of the same rank-order on SPATT samplers as with filtered water: MC-LR, MC-RR and MC-YR by order. The $\%_{var}$ for extracellular MCs was stable along the salinity gradient.

SPATT can represent an efficient warning tool highlighting low water concentrations of extracellular MCs that may occur at early phytoplanktonic bloom development, in case of benthic cyanobacteria, or after bloom collapse. However, these passive samplers only adsorb and cumulate extracellular MCs without release after an episodic and low contamination

event, and do not provide a quantitative representation of environmental concentrations. Moreover, SPATT are not usable for intracellular MCs (in cyanobacteria) that can be predominant during the cyanobacterial blooms. We therefore used two filter-feeding mussel species as an integrating tool of intracellular MCs and of their variation in time through their ingestion of cyanobacteria and dynamic of MC accumulation and elimination.

3.2. Ability of *A. anatina* to reveal the presence of MC producers at freshwater sites

3.2.1. Dynamics of MCs in freshwater phytoplankton and bivalves

At freshwater sites, cyanobacteria dominated the phytoplankton communities in summer and autumn of 2016 and 2017 (density >10,000 cells/mL with a maximum of 2 million cells/mL), with 27 species, among which species of the *Microcystis* genus were most abundant (Bormans et al. 2019). The concentrations of MCs in phytoplankton (intracellular) and in digestive gland tissues (free MCs) from caged *A. anatina* are presented in **Fig. 4**, with a selection of pertinent dates between August 2016 and October 2017. As intracellular MCs were not measured between November 2016 and May 2017, we considered two different periods to evaluate the frequency of occurrence (FO) of MCs in phytoplankton and in bivalves (**Table 1**). At the F1 site, intracellular MCs were measured from August to October 2016, with a maximum of 37 µg/L, and from June to October 2017, with a maximum of 57 µg/L. During these two periods, the bivalves accumulated free MCs, with a FO of 100% and a maximum concentration of 3764 ng/g FW in June 2017 (**Fig. 4, Table 1**). At the F2 site, intracellular MCs were quantified from August to October 2016, with a maximum of 5.9 µg/L, and from June to October 2017, with a maximum of 14 µg/L. During these two periods, the FO of MCs in bivalves was 100%, with a maximum of 3782 ng/g FW in September 2016 (**Fig. 4, Table 1**). These results are in line with previous findings demonstrating that *Anodonta sp.* ingest either colonial (i.e., *Microcystis*) or filamentous (i.e., *Planktothrix*) cyanobacteria, and subsequently

accumulate MCs (Gkelis et al., 2006; Dionisio Pires et al., 2007; Barda et al., 2015; Bontes et al., 2016; Lauceri et al., 2017; Lepoutre et al., 2020). In this study, MC accumulation levels varied depending on which genus dominated the phytoplankton community. The MC levels in mussels were lower when *Planktothrix agardhii* dominated in September 2016 (with 35 µg/L intracellular MCs), and higher when *M. viridis* and *M. aeruginosa* dominated in October 2016 and June 2017 (with 37 and 42 µg/L intracellular MCs, respectively), as also reported by Barda et al. (2015). Nevertheless, *A. anatina* accumulated large amounts of MCs when the environmental contamination was high. A study in the Gulf of Riga, Latvia, showed that *A. anatina* accumulated the greatest amount of MCs (from 0.5 to 88 µg/g DW) among various organisms including fish, gastropods and other bivalves (Barda et al., 2015). Our previous laboratory investigations with *A. anatina* and the freshwater mussel *Dreissena polymorpha* also demonstrated this result, as well as the ability of *A. anatina* to ingest cyanobacteria, accumulate MCs and reveal an environmental contamination as low as 1 µg/L (Lepoutre et al., 2020). This ability of *A. anatina* to indicate a low level of MC contamination was demonstrated in the present *in situ* study in early 2017. Bivalves continuously showed MCs in their digestive glands during the winter period, except in December 2016, suggesting an elimination of MCs previously accumulated, and a *de novo* accumulation in the first month of 2017 when a low cyanobacteria density was observed (1000 cells/mL) (Bormans et al., 2019). *A. anatina* then represents a sensitive bioindicator for environmental monitoring, which integrates intracellular MCs over time between sampling dates of phytoplankton, including low and high contamination levels.

3.2.2. MC variant profiles in phytoplankton and bivalves of freshwater sites

Among the nine MC variants, only MC-LA was never detected in the freshwater sites. The five lowest occurring variants, dmMC-RR, dmMC-LR, MC-LY, MC-LW, MC-LF,

represented 1% or less of the MC concentration in phytoplankton and less than 5% in mussels (**Fig. 3B**). At freshwater sites, the qualitative MC profiles in phytoplankton and mussels differed slightly from those of filtered water and SPATT samplers. The three dominant variants in cyanobacterial samples were MC-RR ($\%_{var}$ from 51 to 55%), MC-LR ($\%_{var}$ from 39 to 43%), and MC-YR ($\%_{var}$ 4%), as already reported (Vasas et al. 2010; Kim et al., 2021). The same MC profile was observed in mussels at both sites (%var from 64 to 80% for MC-RR, from 10 to 19% for MC-LR and from 5 to 7% for MC-YR), but with a significant (chi-squared, $p< 0.05$) increase for MC-RR or decrease for MC-LR in mussels compared to phytoplankton (**Fig. 3B**). This suggests that *A. anatina* accumulated a higher proportion of MC-RR and a lower proportion of MC-LR in their tissues than the amount available in their food, probably due to differential absorption/distribution or metabolization. In contrast, Gupta et al. (2003) reported that MC-LR, MC-YR and MC-RR were highly toxic to mice but that MC-LR had the most severe effects. Detoxification of MC-RR is more efficient than MC-LR in rodents, but the differences are much smaller in human samples (Buratti and Testai 2015). The different hydrophilicities of MC variants may influence their respective bioavailability and propensity for bioaccumulation (McCord et al., 2018). To date, the regulatory guidelines are based on data on MC-LR toxicity because of a lack of toxicological data concerning other variants for which no standards were available. As the amount of purified MC standards has increased recently, differences in toxicokinetics among the most common MC variants have to be investigated.

3.3. Ability of *M. edulis* to reflect MC transfer to estuarine sites

3.3.1. Dynamics of MCs in phytoplankton and bivalves at estuarine sites

The cyanobacteria transfer to the estuary was observed with a progressive decrease in biomass, frequency of occurrence, and species diversity (Bormans et al., 2019). At the first

estuarine site, intracellular MCs were quantified from August to October 2016 and from June to October 2017, with a maximum concentration of 5.9 µg/L and a FO of 86%. During these two periods, the bivalves accumulated MCs with a higher FO (100%) than in phytoplankton samples. Maximum concentrations in *M. edulis* at E1 were 1482 ng/g FW in September 2016 and 447 ng/g FW in September 2017 (**Fig. 4, Table 1**). At the harbour site E2, intracellular MCs were quantified only occasionally in phytoplankton with FO from 31 to 42%, and a maximum concentration of 0.07 µg/L. The FOs of MCs in bivalves were higher than in phytoplankton samples, 100% and 50% in 2016 and 2017, respectively, with maximum MC content of 544 ng/g FW in October 2016 and 166 ng/g FW in June 2017 (**Fig. 4, Table 1**). Finally, at the marine site M, MCs were not detected between August and October 2016, and in only 14% of phytoplankton samples between June and October 2017, with a maximum concentration of 0.03 µg/L. During these two periods, the FOs of free MCs in mussel tissues were 0 and 15% (respectively for 2016 and 2017 periods) with a maximum MC concentration of 22.6 ng/g FW.

We observed a gradual decrease in MC occurrence and accumulation levels from upstream to downstream, probably resulting from the sequential filtration of cyanobacteria flooding from upstream by *M. edulis*, according to the daily rhythm of the tides. Nevertheless, the FO of MCs in *M. edulis* was higher than in phytoplankton samples, demonstrating their ability to integrate environmental MCs between two water samplings, and to mark their transfer from freshwater to estuarine and marine sites. *M. edulis* and *M. trossulus* have already demonstrated their ability to reveal an environmental MC contamination more efficiently than intermittent water samplings (Pobel et al., 2011; Preece et al., 2015).

No quantification of intracellular MCs was performed from November 2016 to April 2017 on phytoplankton samples, but blue mussels still showed MC content (mean of 37 ± 15 ng/g, 47 ± 29 ng/g, and 19 ng/g at the E1, E2 and M sites respectively) in their tissues during the winter period. At E1, low densities (around 100 cell/mL) of cyanobacteria were routinely

detected, with a dominance of *M. viridis* (November), *M. aeruginosa* (January), *P. agardhii* (February) and *Aphanizomenon sp* (April) (Bormans et al., 2019). Similarly, at E2, a cyanobacterial density of 80 cell/mL was observed in January. At the marine site, no cyanobacteria were detected in water samplings during winter, but *M. edulis* sporadically accumulated MCs (after a period without MCs in its tissues) demonstrating its sensitivity as a bioindicator of low environmental contamination levels.

3.3.2. MC variant profiles in phytoplankton and bivalves of estuarine sites

MC-LF and MC-LA were not detected at the estuarine sites, in contrast with what was reported in the Curonian Lagoon, Southern Baltic Sea (Overlingė et al., 2020) and in the freshwater-marine continuum of Puget Sound, Washington (Preece et al., 2015). The variants dmMC-LR, MC-LY, and MC-LW, represented < 1% of the MC concentration in phytoplankton and mussel samples at E1 and completely disappeared at E2 and M (**Fig. 3B**). The rank-order of the three dominant MC variants in phytoplankton samples was slightly changed along the salinity gradient: in order MC-LR ($\%_{var}$ from 53 to 56%), MC-RR ($\%_{var}$ from 29 to 38%), and MC-YR ($\%_{var}$ from 5 to 16%) at E1 and E2 sites, and MC-RR ($\%_{var}$ 67%), MC-LR ($\%_{var}$ 32%) and dmMC-RR ($\%_{var}$ 1.62%) at site M. The mean $\%_{var}$ of MC-RR in phytoplankton increased along the salinity gradient from E1 to M, whereas the mean $\%_{var}$ of MC-YR decreased up to total disappearance from sample. These changes in variant profiles can be attributed to the selection of cyanobacteria species and strains along the salinity gradient (Bormans et al., 2019). The $\%_{var}$ of MC-YR was similar between phytoplankton and mussel tissues, and decreased in mussel tissues along the salinity gradient from 13% at E1 to 0% at M. The MC-LR variant showed a similar and stable $\%_{var}$ between mussel and phytoplankton samples at the three estuarine sites, suggesting that *M. edulis* accumulated MC-LR and MC-YR in proportions similar to what they ingest. This observation is in

contradiction to the lower MC-LR ratio observed in mussel tissues compared to cyanobacteria at freshwater sites of this study and in the laboratory exposure of *M. edulis* to *M. aeruginosa* (Camacho et al., 2021). While the MC-RR variant occurred more frequently in tissues of *A. anatina* than in phytoplankton at freshwater sites, the contrary was observed at estuarine sites where the $\%_{var}$ of MC-RR decreased in mussels along the salinity gradients (from 42% at E1 to 6% at M) despite a concomitant increase in phytoplankton (from 28% at E1 to 66% at M). This suggests either that *M. edulis* accumulates less MC-RR as the salinity increases, as a result of changes in metabolic processes, or also filter MCs dissolved in water (extracellular) or adsorbed on organic or inorganic particles. However, we did not observe a change in the proportion of extracellular MC-RR along the salinity gradient. Moreover, the proportion of MC-YR was similar between phytoplankton and mussel tissues, both decreasing along the salinity gradient, and was stable in water and on SPATT samplers (extracellular) along the freshwater-marine continuum. This reinforces the hypothesis that contamination of mussels with MCs occurs mainly through the filtration of cyanobacteria flowing downstream with differential accumulation kinetics between variants, probably influenced by environmental parameters such as the salinity; a hypothesis that should be further assessed. Similarly, Kim et al. (2019; 2021) showed that the concentration of MC-RR was lower than MC-LR and MC-YR in clams, worms, crabs, and fish from the Geum river estuary in Korea. Interestingly, in the present study, marine mussels accumulated less MC-RR but more dmMC-RR than was available in food. The proportion of dmMC-RR significantly increased in mussels (from 3 to 50%) when the salinity increased, despite little change (1 to 2%) in phytoplankton. One could hypothesize metabolism of MC-RR, abundant in phytoplankton, to dmMC-RR, in mussel tissues, but this is unlikely. Lastly, *M. edulis* may also accumulate MCs from sediment, which could have a different MC variant profile than the one in cyanobacteria flowing from upstream. This could explain the presence of extracellular MC-YR at estuarine and marine sites, with lower levels of intracellular MC-YR. A recent study reported that sediments from a

river delta contained MCs, even during periods that were not typical proliferation months (Bolotaolo et al., 2020). These MCs may originate from planktonic cyanobacteria sinking onto the sediments or from benthic MC-producing cyanobacteria. In a joint survey, we reported the presence of intact colonies of *Microcystis* with *mcyB* genes and of intracellular MCs in the sediment at the E2 site (harbour) (Bormans et al., 2020). MCs in sediment may also originate from extracellular MCs contained in or adsorbed onto particles that are periodically released into the water during a rising tide (Henao et al., 2020). The periodic adsorption and release of different MC variants from sediment probably varies according to their respective partitioning coefficient, which is influenced by water pH (McCord et al., 2018).

3.4. Free and total MC accumulation in *M. edulis* and risk for consumers

The potent risk for human was evaluated using data on MC accumulation in *M. edulis* sampled at the harbour and marine sites where mussels collection for human consumption is realistic. Mean free MC contents in whole *M. edulis* (15.7 ± 6.8 ng/g FW at E2, 1.9 ± 0.8 ng/g FW at M) were in agreement with values (6.5 ng/g) reported for *M. trossulus* by Preece et al. (2015) but lower than the ones reported for *M. galloprovincialis* from the Adriatic Sea, Italy (1.7-256 ng/g) and from the Amvrakikos Gulf, Greece (45-142 ng/g) (De Pace et al., 2014 ; Vareli et al., 2012). We calculated the safe MC threshold concentration in mussels based on the WHO TDI of 40 ng MCs/day/kg of body weight, a standard body weight of 70 kg, and a mean daily intake of fresh shellfish for the population of 27 and 77 g/day for average and high consumers, respectively (ANSES, 2017; Rodríguez-Hernández et al., 2019). The threshold MC concentrations in mussels were 103 and 36 ng/g FW for average and high consumers, respectively. At the harbour site E2, concentrations of free MCs in *M. edulis* only exceeded the threshold for high consumers on two occasions in October and December 2016 (**Fig. 5**),

and were therefore not sufficient to provoke adverse human health effects with respect to the provisional WHO TDI. However, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) recently revised the sub-chronic exposure criteria value based on a study of the reprotoxic effects of MC-LR on rodents (Chen et al., 2011), and proposed a reference value of 1 ng MCs/day/kg BW (ANSES, 2020). Based on this updated value, the threshold MC concentration in mussels for average and high consumers (2.6 and 0.9 ng/g FW) would have been exceeded during 6 and 8 months, respectively, in mussels from the harbour site from September 2016 to September 2017 (**Fig. 5**). Mussels from the marine site close to farming areas exceeded the threshold MC concentration for high consumers on two dates. The consumption of mussels from the harbour over a period of several weeks would be sufficient to expose consumers to potent reprotoxic effects described in rodents (decreased sperm count and mobility, increase in sperm anomalies). However, when consumption exceeds the recommendations, the potent sub-chronic effects are unlikely to be diagnosed because of a lack of specific diagnostic criteria and of knowledge of the risk by health practitioners. These poisonings are likely to occur during summer and autumn from mussels farmed or collected close to the tideline at sites receiving flows of fresh waters that experience cyanobacterial blooms. Mussel consumers might also be co-exposed to other freshwater cyanotoxins and to marine phycotoxins such as domoic acid and brevetoxins (Peacock et al., 2018; Metcalf et al., 2020).

Previous studies demonstrated that from 60 to 90% of total (free plus protein-bound) MCs exist in protein-bound form in mollusc tissues, and that protein-bound MCs remained longer in tissues than the free MCs (Lance et al., 2010; Pham et al., 2015; Lepoutre et al., 2020). At the freshwater sites, total MC content in *A. anatina* was significantly higher than free MC (Mann-Whitney Test, $p < 0.01$), but the FO of positive samples was similar (**Fig. 6**). At estuarine and marine sites, both the total MC contents in mussels and the FO of positive samples were higher than for free MCs (Mann-Whitney Test, $p < 0.01$). Measuring the total

MCs allowed us to highlight an accumulation at the marine site for the three investigation dates at which no free MCs were quantified. This suggests that the frequency of MCs in mussels at marine sites could have been far higher than 14% if we had quantified total MCs at every sampling date. If considering total MC content, the threshold MC concentration in mussels for average and high consumers based on WHO and French provisional guideline values would have been exceeded at each sampling date. However, the toxicity of protein-bound MCs for consumers has not been investigated so far. If MCs bound to proteins remain toxic, become free through release by proteolytic enzymes, or are metabolized as a toxic compound during the digestive process, then measuring only free MCs in seafood may underestimate the risk for consumers. While awaiting data on the bioavailability, bioaccessibility, and toxicity of total MCs, their analysis in seafood may improve available data for future mussel consumption advisories.

4. Conclusions

Our study suggests that the bivalves *A. anatina* and *M. edulis* represent useful integrative tools for monitoring the contamination of fresh, estuarine and marine waters by MCs. While SPATT only collect extracellular MCs and are passive cumulative samplers, they remain a complementary tool to bivalve especially to highlight low MC concentrations. Globally, the qualitative profiles of MC variants were similar between phytoplankton and mussels, and between filtered waters and SPATT samplers, with a dominance of MC-LR, -RR and -YR, and with some differences in variant accumulation capacities by mussels. Marine mussels were better indicators of MC transfer from fresh to estuarine waters than were monthly water samplings. Compared to intermittent water samplings, using SPATT or bivalves could give a fairer view of the contamination level of a freshwater-marine continuum, in which the downstream transfer of cyanobacteria and cyanotoxins can be episodic. This study is the first

to report the contamination of marine mussels *M. edulis* by cyanotoxins at a French estuarine site. Based on the updated French sanitary guideline values, the level of free MCs in *M. edulis* tissues may present a potent risk for people who regularly consume mussels. The level of total (free and protein-bound) MCs in mussel tissues may be of greater concern, but the use of total MC content in health risk assessment is still being debated as no data on their toxicity are available. Our results demonstrate the importance of including both free and total MCs in national monitoring programs for marine food originating from estuarine zones, and for health practitioners to improve reporting of cases of cyanotoxin poisoning and related symptoms.

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This article is dedicated to the memory of our dear friend and colleague Yves Le Medec.

Declaration of interest

Herewith we affirm that:

- All authors have seen and approved the submission of the manuscript.
- There is no related work in review or press.
- The manuscript has been submitted exclusively to WATER RESEARCH.
- None of the authors has a conflict of interests related to the manuscript.

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Figure 1. Map of the freshwater (F1, F2), estuarine (E1, E2) and marine (M) sites, with the two caged bivalves species (*A. anatina* in F1 and F2, and *M. edulis* in E1, E2 and M), during the two-year investigation period.

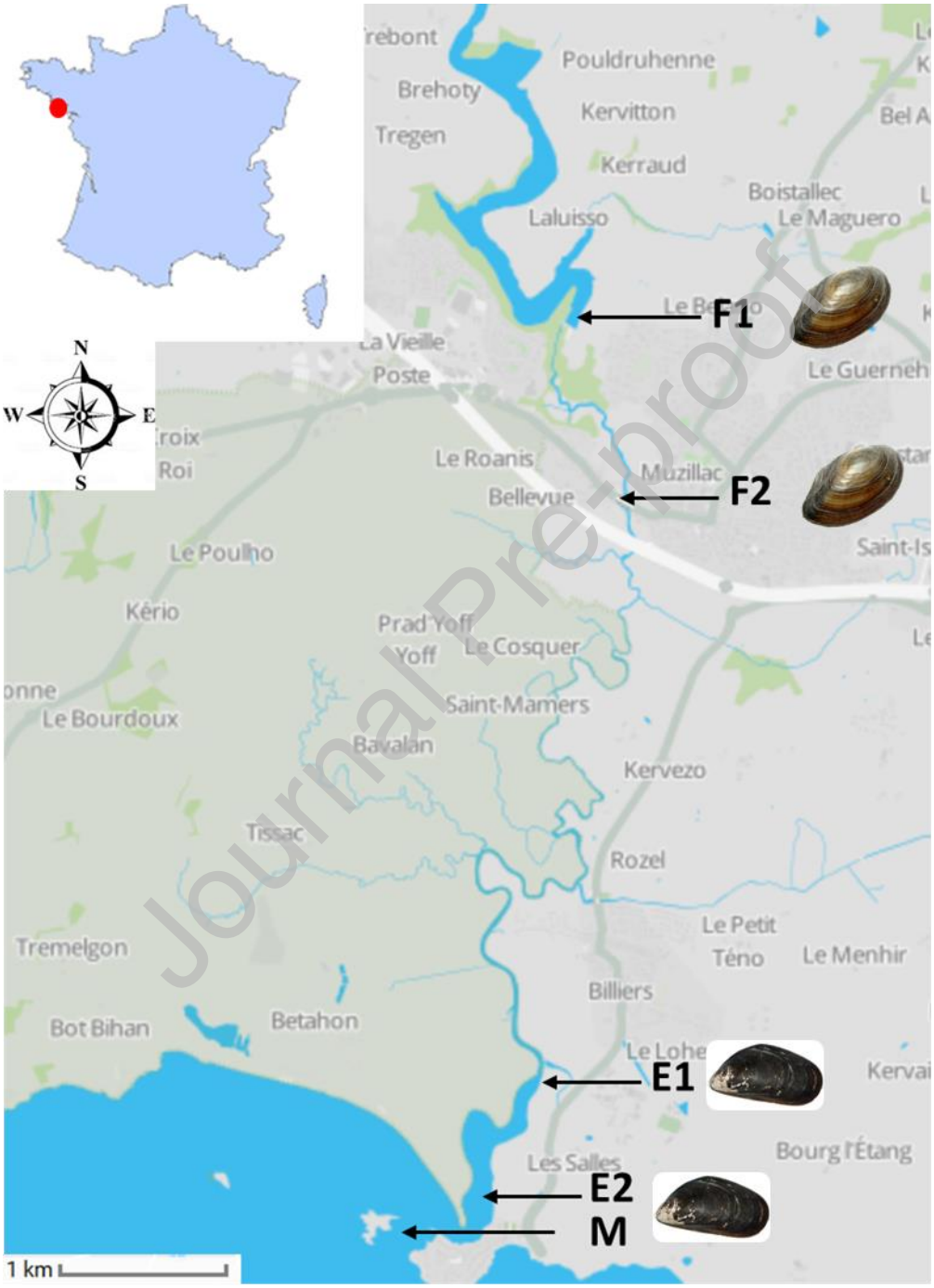


Figure 2. Concentration of MCs (sum of the nine MC variants dmMC-RR, MC-RR, MC-YR, MC-LR, dmMC-LR, MC-LA, MC-LY, MC-LW, MC-LF) measured in filtered water (extracellular MCs in µg/L, bar chart) and in SPATT samplers (amount of MCs adsorbed in the membranes during the deployment expressed in ng/g of resin/day, areas), from July to October 2017 at the freshwater (F1, F2), estuarine (E1, E2) and marine (M) sites. ND: Not Determined.

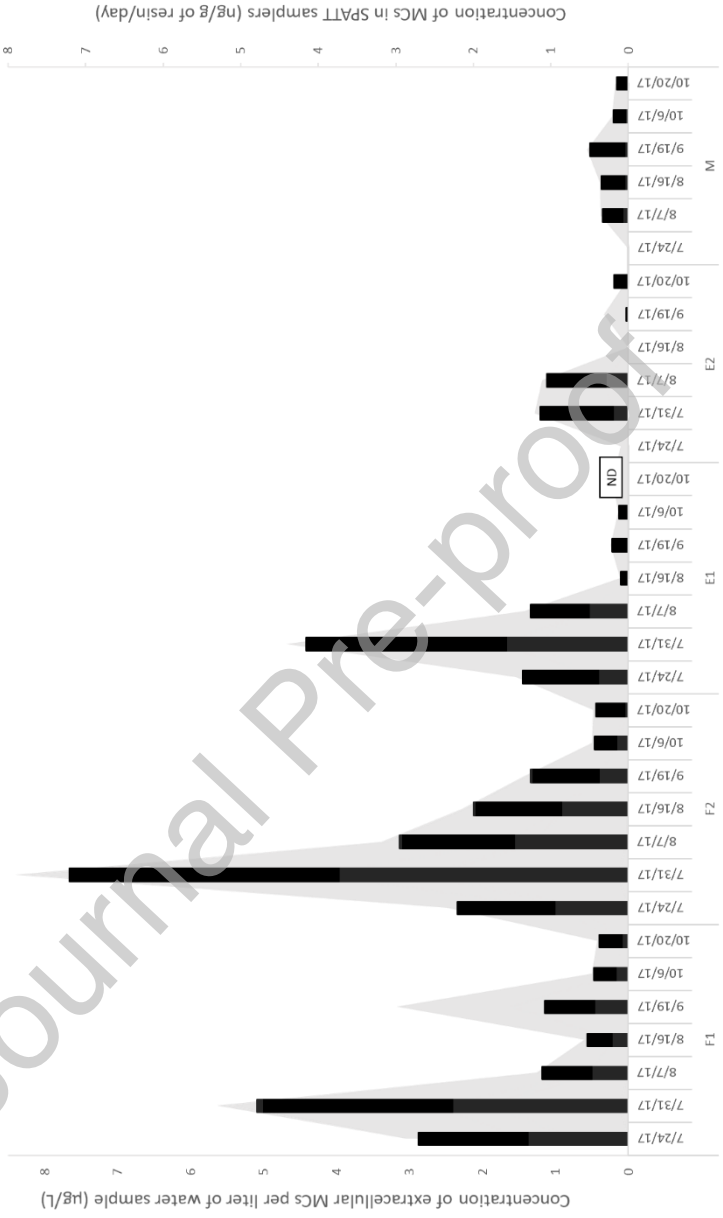


Figure 3: Mean percentages of the nine MC variants (dmMC-RR, MC-RR, MC-YR, MC-LR, dmMC-LR, MC-LA, MC-LY, MC-LW, MC-LF) in A) filtered water (extracellular MCs) and SPATT samplers (extracellular MCs) from July to October 2017, and B) phytoplankton samples (intracellular MCs) and tissues of caged mussels *A. anatina* and *M. edulis* (free accumulated MCs) from August 2016 to October 2017.

(color should be used for this figure)

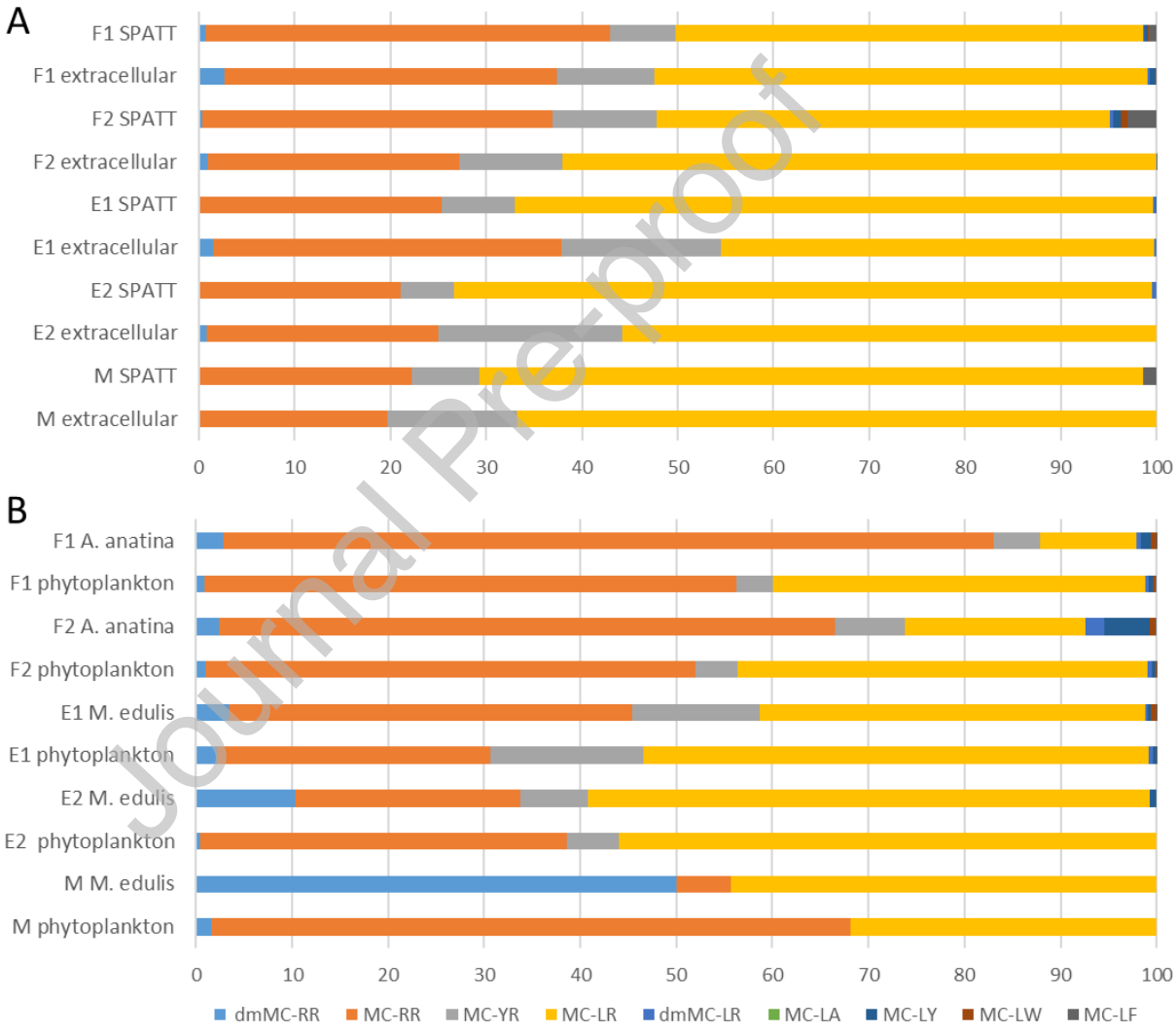


Figure 4: Concentration of MCs (sum of the nine MC variants) measured in phytoplankton (intracellular in µg/L, bar chart) and in caged mussels (free/non-protein bound accumulation fraction in *A. anatina* and *M. edulis* in ng/g FW, areas) sampled from August 2016 to October 2017 (selected dates are presented here) at the freshwater (F1, F2), estuarine (E1, E2) and marine (M) sites. Value scales in Y axis changes among sites. ND: Not Determined.

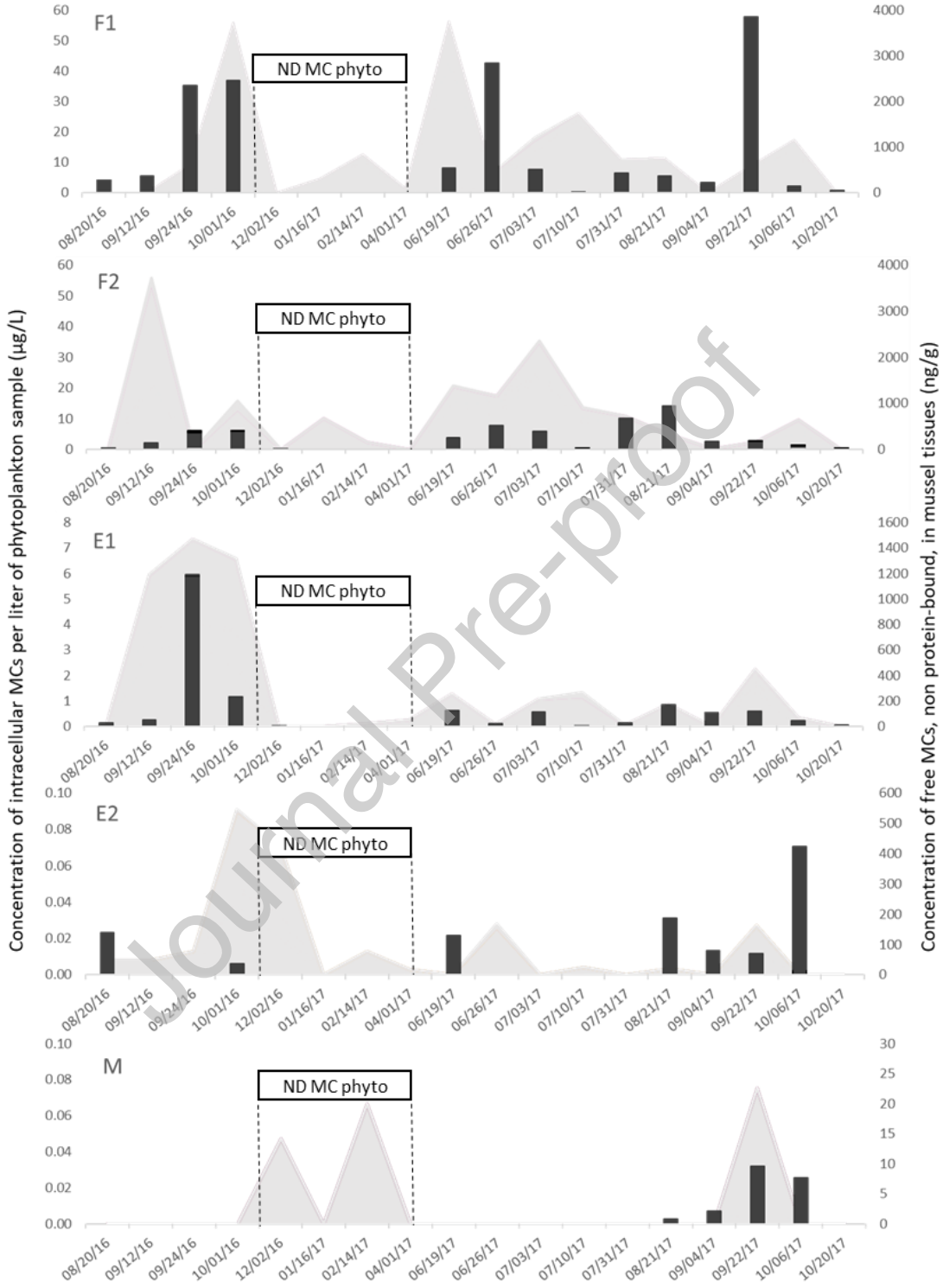


Figure 5: Free MC concentrations (in ng/g FW) in whole *M. edulis* from September 2016 to September 2017 at the harbour site E2 (grey bar chart) and the marine site M (white bar chart). The safe MC threshold concentrations in mussels for human were evaluated for average (black full dash) or high (black dotted dash) consumers based on the new French guideline value of ANSES, and for average (not visible, value of 103 ng/g FW which extends beyond the scale of the y-axis) or high (grey dotted dash) consumers based on the WHO TDI.

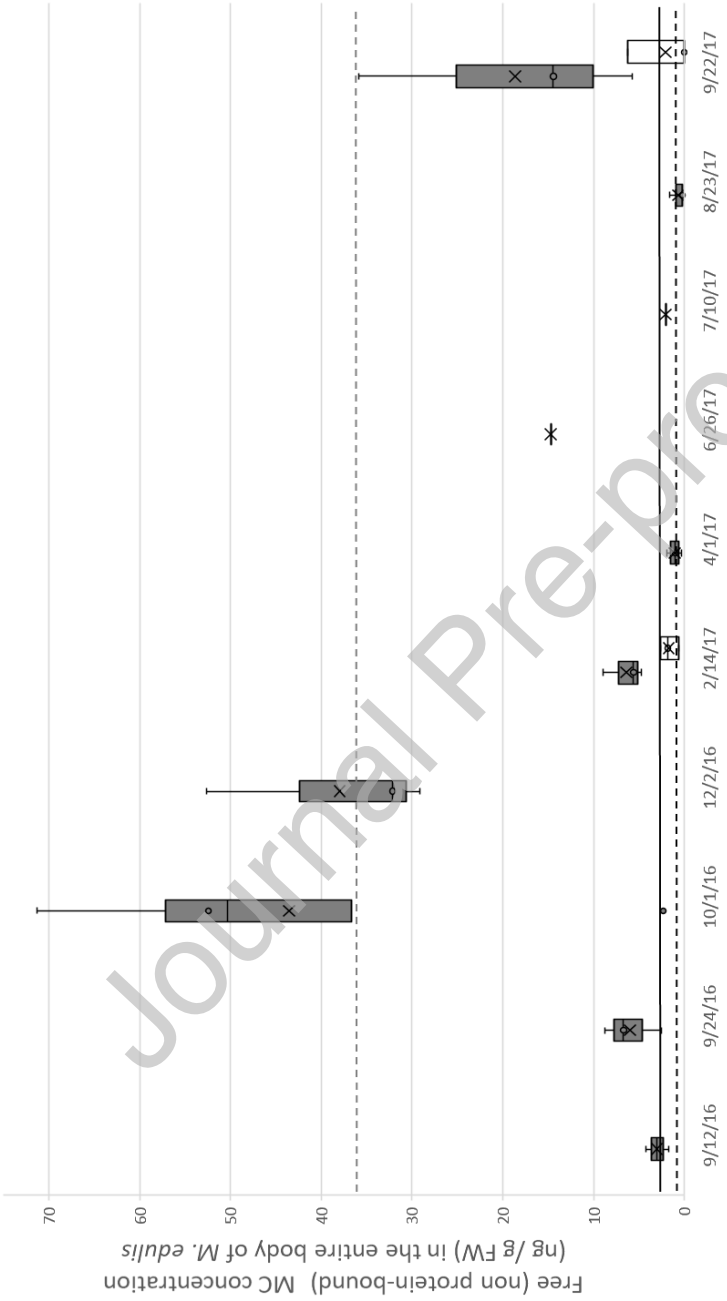
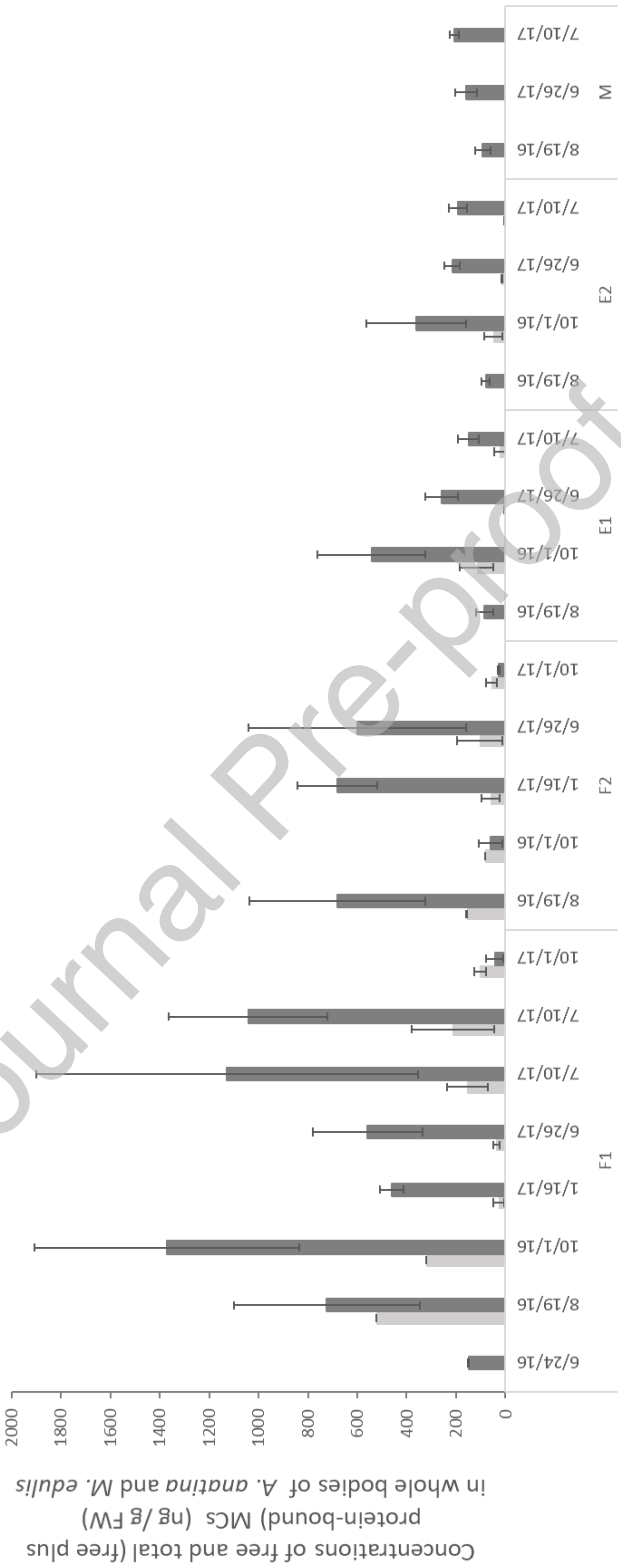
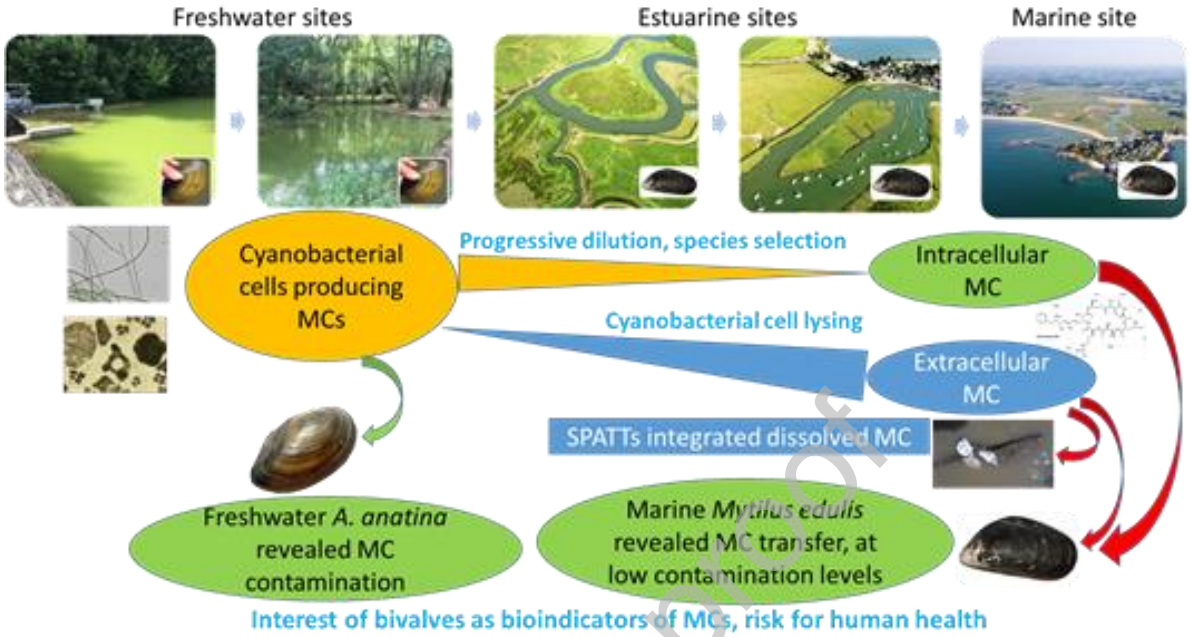


Figure 6: Free (light grey color) and total (including free plus protein-bound, dark grey color) MC concentrations (in ng/g FW) in whole bodies of some individuals of *A. anatina* (F1 and F2 sites) and *M. edulis* (E1, E2 and M sites) from June 2016 to October 2017.





Graphical

Table 1: Frequency of occurrence (FO in %) and mean + SD positive concentrations of MCs in phytoplankton biomass (intracellular) and in tissues of freshwater and marine bivalves (free MCs = are not protein-bound), from August to October 2016 and from June to October 2017. The FO and mean + SD concentrations of intracellular MCs (in phytoplankton) are indicated in brackets.

Sites		FO (%) MCs in bivalves (FO MCs in phytoplankton)		Mean+SD positive free MCs concentration (ng/g FW) in bivalves	
		2016	**2017**	**2016**	**2017**
F1	*A. anatina*	**100** (100)	**100** (100)	4.1 ± 1.8	1056,5 ± 440.1
F2	*A. anatina*	**100** (100)	**100** (100)	946.9 ± 644.9	1002.7 ± 331.6
E1	*M. edulis*	**100** (86)	**100** (87)	996,0 ± 336.9	181.1 ± 60.6
E2	*M. edulis*	**100** (42)	**50** (31)	223.1 ± 139.5	93.1 ± 33.9
M	*M. edulis*	**0** (0)	**15** (14)	0	22.6